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INVENTOR: Ebringer

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**DECLARATION OF ALAN EBRINGER UNDER 37 CFR §1.132**

I, Alan Ebringer, the sole inventor named in the above-identified patent application and the sole inventor of the subject matter claimed therein, do hereby declare and state as follows:

1. As the sole named inventor of the above-identified patent application, I am intimately familiar with the contents of the application and the subject matter claimed therein. I am the sole inventor of the subject matter disclosed and claimed in the above-identified patent application.

2. I am currently employed as a Professor of Immunology, at King's College London, London, England. I am also currently an Honorary Consultant Rheumatologist at the Middlesex Hospital, University College Hospital School of Medicine, London. I hold an M.D. degree, as well as a B.Sc. degree. I am a Fellow of the Royal College of Physicians-UK (FRCP), a Fellow of the Royal Australasian College of Physicians (FRACP), and a Fellow of the Royal College of Pathologists (FRCPath). I have authored and co-authored numerous papers addressing the etiology, diagnosis, transmission, and treatment of bovine spongiform encephalopathy (BSE).

3. Attached hereto and incorporated herein by reference is a paper that I co-authored entitled "Antibody Responses to *Acinetobacter* spp. and *Pseudomonas aeruginosa* in Multiple Sclerosis: Prospects for Diagnosis Using the Myelin-*Acinetobacter*-Neurofilament Antibody Index." The paper appeared in the journal *Clinical and Diagnostic Laboratory Immunology*, November 2001, pages 1181-1188. I am the corresponding author of the paper.

4. All of the experiments presented in the attached paper were performed either personally by me or at my direction and under my supervision.

5. The experiments presented in the attached paper were conducted to show, in part, that an elevated level of antibodies specific to *Acinetobacter* spp. in a human subject, as compared to the corresponding antibody level in a control human population, is indicative of multiple sclerosis in the human subject tested.

6. To test the above hypothesis, sera from 26 multiple sclerosis (MS) patients (the human test subject) were obtained. Diagnosis of MS was made according to the Poser criteria, a widely accepted means of diagnosing MS. Sera from 25 normal human subjects (the control group) were also obtained.

7. Cultures of *Acinetobacter* sp. strain 11171, *Acinetobacter* sp. strain 19004, *Acinetobacter junii* 17908, *Acinetobacter lwoffii* 5866, and *Acinetobacter radioresistens* (sp. 12) were obtained from the Public Health Laboratories, Nottingham, UK. *Acinetobacter calcoaceticus* (NCIMB 16904) was obtained from the National Collections of Industrial and Murine Bacteria, Ltd., Aberdeen, Scotland. The cultures were grown in 1 L flasks on an orbital shaker for 2 days at 30°C, in 200 mL of nutrient broth. Flasks were inoculated with two loopfuls of starter culture and left shaking for 6 hours at 37°C. Batch cultures were harvested by centrifugation. Pellets of cells were washed three times in 0.15 M PBS (pH 7.4) and resuspended in 10 mL of PBS. For the ELISA described in paragraph 8 herein, a stock solution of each bacterial suspension was prepared by diluting the suspension in 0.05 carbonate buffer (pH 9.6) to give an OD reading of 0.25 at 540 nm. For SDS-PAGE, the resuspended pellet was ultrasonicated at an amplitude of 12  $\mu$ m with 30-second bursts and 60-second rest periods (7 cycles). The protein content of the sonicated samples was measured using Bradford's protein assay. The samples were then diluted in sample buffer (0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.001% bromophenol blue) to a protein concentration of 1  $\mu$ g/ $\mu$ L and heated at 100°C for 3 minutes.

8. Enzyme-linked immunosorbent assays (ELISAs) were prepared by taking aliquots of 200  $\mu$ L of bacterial suspension, or bovine myelin basic protein (MBP), or neurofilaments (25  $\mu$ g/mL, Sigma), diluted in a 0.05 M carbonate buffer (pH 9.6), and adsorbing these onto a 96-well flat-bottom polystyrene microELISA plate (Dynatech) overnight at 4°C. Plates were washed three times for 5 min. in PBS containing 0.05% wt/vol Tween 20 ("washing" and "incubation" buffer) and were then blocked with PBS containing 0.1% BSA for 1 hr at 37°C. The washing procedure was repeated and 200  $\mu$ L of control or test serum, diluted 1 in 200 in incubation buffer, was added to the wells in duplicate and incubated for 1 hr at 37°C. Plates were washed three times in washing buffer, and 200  $\mu$ L of IgM, IgG, or IgA rabbit anti-human conjugate with horseradish peroxidase (diluted 1 in 500 with incubation buffer) was added and incubated for 1 hr at 37°C. The washing procedure was repeated, and 200  $\mu$ L of substrate solution was added (substrate solution = 0.5 mg/mL 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in citrate phosphate buffer, pH 4.1, containing 0.98 mM H<sub>2</sub>O<sub>2</sub>). The plates were developed in the dark, at room temperature, for 25 min. The reactions were stopped by adding 100  $\mu$ L of sodium fluoride (2 mg/mL). Absorbances were measured on a microtiter plate reader (Dynatech MR606) at 630 nm. All studies were carried out under blind conditions where the tester did not know which sera were test samples and which sera were controls.

9. The mean OD units of control groups were compared with the mean OD of the 26 MS patients using a one-tail Student's *t*-test, and 95% confidence limits of control groups were calculated. Pearson's correlation coefficient (*r*) was also calculated using the statistical package Prism 3.0 (GraphPad Software).

10. The results of the ELISAs described in paragraph 8 were as follows:

A. Levels of IgA antibodies to *Acinetobacter* sp. strain 11171 ( $P < 0.0001$ ), *Acinetobacter* sp. strain 19004 ( $P < 0.0001$ ), *Acinetobacter junii* 17908 ( $P < 0.01$ ), *Acinetobacter lwoffii* 5866 ( $P < 0.0001$ ), and *Acinetobacter radioresistens* ( $P < 0.0001$ ) in MS patients were significantly higher than those in the healthy control group.

B. Levels of IgG antibodies to *Acinetobacter* sp. strain 11171 ( $P < 0.0001$ ), *Acinetobacter* sp. strain 19004 ( $P < 0.0001$ ), *Acinetobacter junii* 17908 ( $P < 0.0001$ ), *Acinetobacter lwoffii* 5866 ( $P < 0.0001$ ), and *Acinetobacter radioresistens* ( $P < 0.0001$ ) in MS patients were significantly higher than those in the healthy control group.

C. Levels of IgM antibodies to *Acinetobacter* sp. strain 11171 ( $P < 0.0001$ ), *Acinetobacter* sp. strain 19004 ( $P < 0.0001$ ), *Acinetobacter junii* 17908 ( $P < 0.0001$ ), *Acinetobacter lwoffii* 5866 ( $P < 0.0001$ ), and *Acinetobacter radioresistens* ( $P < 0.0001$ ) in MS patients were significantly higher than those in the healthy control group.

11. The results presented in paragraph 10, and in the paper attached hereto and incorporated herein by reference, clearly show, by way of objective scientific evidence, that an elevated level of antibodies specific to microorganisms of the genus *Acinetobacter* in a human test subject as compared to the corresponding level in known normal individuals, is indicative of MS in the test subject.

12. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this Rule 132 Declaration is directed.

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Alan Ebringer

Date